Supplementary data

iTRAQ-based Proteomics Analyses

Protein preparation and digestion. P. aeruginosa strains were grown in in AB minimal medium supplemented with 5g L^{-1} glucose for 48 hours at 37 °C with shaking. After harvesting, cell pellet was washed with 1X PBS and resuspended in 2 ml of lysis buffer containing 0.5M TEAB, 0.1M SDS and protease inhibitor cocktails (Sigma-Aldrich). The cells were ruptured by sonication, and the cell debris was removed by centrifugation at 4 °C at 16000 × g for 15 min. Three biological replicates for each growth conditions were pooled together and 200 µg of proteins from each growth condition were dissolved in equal volume of sample buffer (Invitrogen) supplemented with 0.5% 2-mercaptoethanol and denatured by boiling at 95 °C for 5 min. 1D gel electrophoresis was carried out using 10% SDS-PAGE for in-gel digestion.

Proteins were first reduced in 5 mM Tris-(2-carboxyethyl) phosphine (TCEP) for 1 h at 60 ºC, followed by blocking cysteine residues in 10 mM methyl methanethiosulfate (MMTS) for 30 min at room temperature in the dark. Trypsin was added at a ratio of 1:50 (trypsin/sample). It was then incubated at 37 ºC overnight. The tryptic peptides were extracted by 50%ACN/5%Acetic Acid from gel for 3 times and were desalted using Sep-Pak C18 cartridges (Waters, Milford, MA) and dried in a SpeedVac (Thermo Electron, Waltham, MA). All chemicals were purchased from Sigma-Aldrich unless stated otherwise.

iTRAQ labeling. The iTRAQ labeling of the tryptic peptides was performed using 4-plex iTRAQ reagent kit (Applied Biosystems, Foster City, CA), according to manufacturer's protocol. 200 µg peptides from each condition were individually labeled with respective isobaric tags (PAO1Δ*wspF* sample with 114, PAO1/pLac::YhjH sample with 115), followed by 2 h

incubation, then quenched by water, desalted using C18 solid phase extraction cartridge, and then vacuum centrifuged to dryness. The iTRAQ labeled peptides were reconstituted in Buffer A (10 mM ammonium acetate, 85% acetonitrile, 0.1% formic acid) and fractionated using ERLIC column (200 x 4.6 mm, 5µm particle size, 200 Å pore size) by HPLC system (Shimadzu, Japan) at flow rate of 1.0 ml/min using our previously optimized protocol (1). The HPLC chromatograms were recorded at 280 nm and fractions were collected online using automated fraction collector. 20 fractions were collected and concentrated using vacuum centrifuge and reconstituted in 3% ACN with 0.1% formic acid for LC-MS/MS analysis.

LC-MS/MS. The peptides were separated and analyzed on a home-packed nanobore C18 column (15 cm x 75 µm; Reprosil-Pur C18-AQ, 3 µm, Dr Maisch, Germany) with a Picofrit nanospray tip (New Objectives, Woburn, MA, USA) on a Tempo^{TM} nano-MDLC system coupled with a QSTAR® Elite Hybrid LC-MS/MS system (Applied Biosystems). Peptides from each fraction were analyzed in triplicate by LC-MS/MS over a gradient of 90 min. The flow rate of the LC system was set to a constant 300 nl/min. Data acquisition in QSTAR Elite was set to positive ion mode using Analyst® QS 2.0 software (Applied Biosystems). MS data was acquired in positive ion mode with a mass range of 300–1600 m/z. Peptides with +2 to +4 charge states were selected for MS/MS. For each MS spectrum, the three most abundant peptides above a five-count threshold were selected for MS/MS and dynamically excluded for 30 s with a mass tolerance of 0.03 Da. Smart information-dependent acquisition was activated with automatic collision energy and automatic MS/MS accumulation. The fragment intensity multiplier was set to 20 and maximum accumulation time was 2 s.

Data analysis. Spectra acquired from the three technical replicates were submitted to ProteinPilot (v3.0.0.0, Applied Biosystems) for peak-list generation, protein identification and

quantification. User defined parameters of the Paragon algorithm in ProteinPilot software were configured as follows: (i) Sample Type, iTRAQ 2-plex (Peptide Labeled); (ii) Cysteine alkylation, MMTS; (iii) Digestion, Trypsin; (iv) Instrument, QSTAR Elite ESI; (v) Special factors, Urea denaturation; (vi) Species, None; (vii) Specify Processing, Quantitate & Bias Correction; (viii) ID Focus, biological modifications, amino acid substitutions; (ix) Database, *Pseudomonas aeruginosa* PAO1 PAO1-UW; (x) Search effort, thorough ID; (xi) Result quality, Unused ProtScore (Conf) >0.05 (10.0%). Default precursor and MS/MS tolerance for QSTAR ESI MS instrument were adopted automatically by the software. For iTRAQ quantitation, the peptide for quantification was automatically selected by Pro Group algorithm to calculate the reporter peak area, error factor (EF) and p-value. The resulting data was auto bias-corrected by build-in ProteinPilot algorithm to get rid of any variations imparted due to the unequal mixing during combining different labeled samples. During bias correction, the software identifies the median average protein ratio and corrects it to unity, and then applies this factor to all quantitation results.

The following cut-offs were used for protein identification: Unused Protein Score of at least 2 (i.e. 99% confidence of identification) and having more than 1 peptide identified. Using a p-value cut-off of 0.05, the abundances of 116 proteins were found to be significantly affected by low intracellular levels of c-di-GMP; the abundance of 44 proteins was upregulated, while the abundance of 72 proteins was down-regulated (shown in Tables 2 and 3 respectively). In our study, upregulation was defined as an abundance value (115:114 score) of at least 2.0, and downregulation was defined as an abundance value (115:114 score) below 0.5.

References:

1. **Hao, P., T. Guo, X. Li, S. S. Adav, J. Yang, M. Wei, and S. K. Sze. 2010. Novel application of electrostatic repulsion-hydrophilic interaction chromatography (ERLIC) in shotgun proteomics: comprehensive profiling of rat kidney proteome. J Proteome Res 9:3520-3526.**

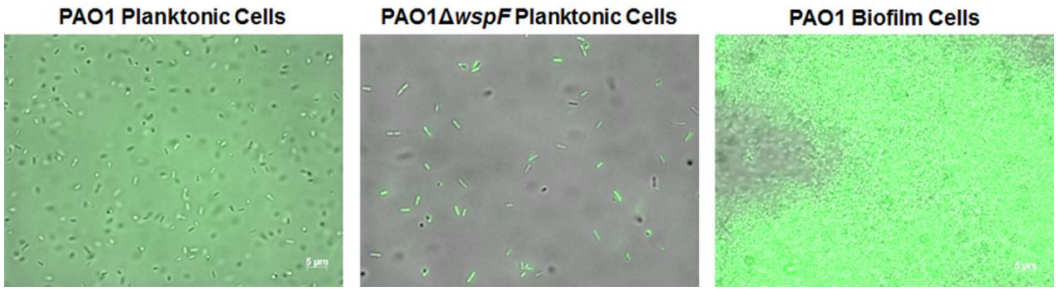

Figure S1. Representative images of p_{cdrA}-*gfp* expression in *P. aeruginosa* PAO1/p_{cdrA}-gfp, PAO1Δ*wspF*/p_{cdrA}-gfp planktonic cells and PAO1 biofilm cells. Three µl cultures of overnight planktonic PAO1/p_{cdrA}-gfp and PAO1Δ*wspF*/p_{cdrA}-gfp cells were spotted onto cover slides for imaging by using a fluorescence microscopy. Slide of PAO1/p_{cdrA}-gfp biofilms was imaged by using a fluorescence microscopy.

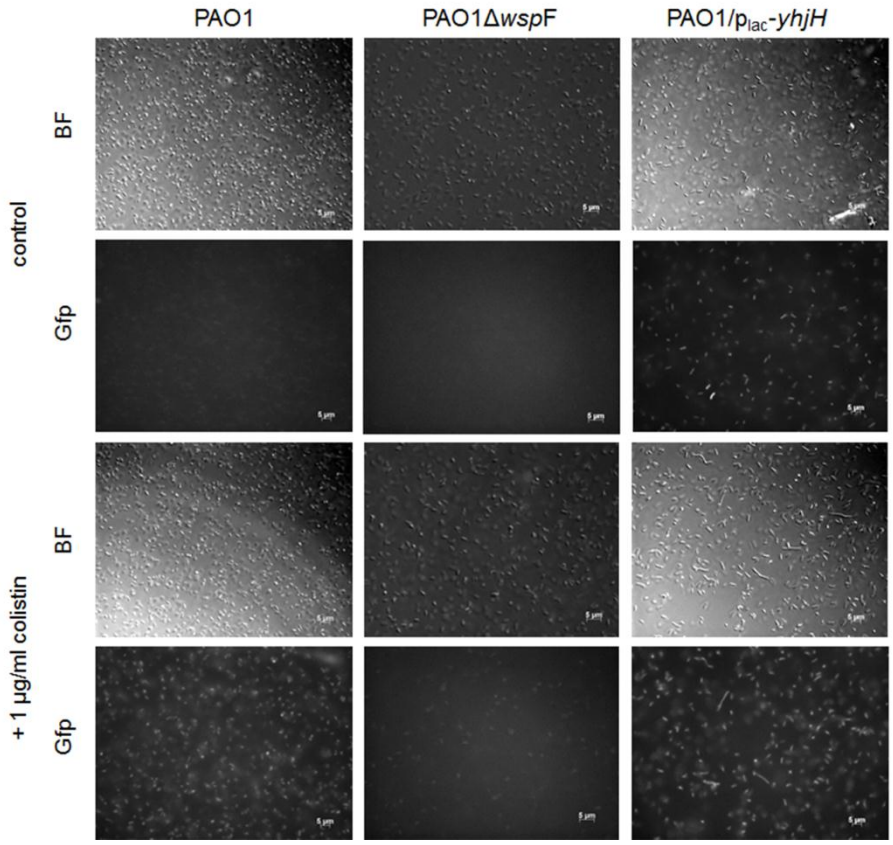

Figure S2. Representative images of p_{pmr}-*gfp* expression in *P. aeruginosa* PAO1 (PCells), PAO1Δ*wspF* (BCells) and PAO1/p_{lac}-*yhjH* (DCells) strains. Overnight cultures were diluted 10 fold into fresh ABTGC medium with and without 1 µg ml^{-1} colistin. 3 µl cultures of each condition were spotted onto cover slides after 7 h growth for imaging by using a fluorescence microscopy.

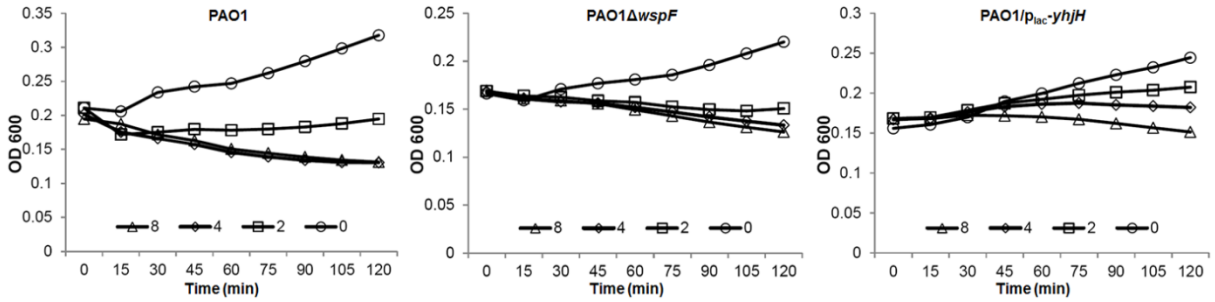

Figure S3. Time-kill kinetics of colistin at 2, 4, 8 µg mL^{-1} final concentrations to *P. aeruginosa* PAO1 (PCells), PAO1Δ*wspF* (BCells) and PAO1/p_{lac}-*yhjH* (DCells) cultures at OD600 = 0.2. Killing kinetics was monitored by using the TECAN Infinite Pro2000 microplate reader. Means of duplicates are shown.